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ORIGINAL RESEARCH

Anti-inflammatory Role of Trilobatin on Lipopolysaccharide-induced Acute Lung Injury through Activation of AMPK/GSK3β-Nrf2 Pathway

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Abstract

Inflammation is essential for the pathological process of acute lung injury (ALI). Trilobatin, a glycosylated dihydrochalcone can show anti-oxidative and anti-inflammation properties. This study aimed to explore whether trilobatin could suppress inflammation in lipopolysaccharide (LPS)-induced ALI. Firstly, mice were injected with trilobatin intraperitoneally, and then LPS was administered intranasally to induce lung injury. Data from analysis of lung edema and pathologic histology of lung tissues indicated that pretreatment with trilobatin alleviated LPS-induced histopathological changes and decreased wet-to-dry weight (W/D) ratio. Moreover, LPS-induced lung injury was attenuated post trilobatin treatment with reduced protein concentration, cell numbers, neutrophils and macrophages in BALF (bronchoalveolar lavage fluid). Secondly, trilobatin treatment decreased the protein level of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) thereby suppressing LPS-induced inflammation. LPS-induced oxidative stress was ameliorated following trilobatin treatment with decreased malondialdehyde (MDA) and increased glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). Lastly, trilobatin decreased NF-κB phosphorylation and increased Nrf2 through up-regulation of AMPK and GSK3β phosphorylation. In conclusion, trilobatin repressed oxidative stress and inflammatory damage by ameliorating LPS-induced ALI through activation of AMPK/GSK3β-Nrf2 and inhibition of NF-κB.

Keywords

Trilobatin, Lipopolysaccharide, Acute lung injury, Oxidative stress, Inflammation, AMPK/GSK3β-Nrf2

1. Introduction

Acute lung injury (ALI) with a high mortality is considered to be an acute, severe inflammatory process in the lungs [1]. ALI is often associated with inflammation and oxidative stress, which could contribute to sepsis-induced ALI [2]. At present, there is devoid of effective pharmacological therapies for ALI [3]. Identification of therapeutic targets that regulate inflammation or oxidative stress might be a potential strategy to prevent an treat ALI.

Flavonoid is a kind of polyphenolic compounds that is widely distributed in the plant kingdom, with a variety of biological properties including antioxidant [4], anticancer [5] and anti-inflammatory [6]. Trilobatin is a glycosylated dihydrochalcone (a type of flavonoid) extracted from the *Lithocarpus polystachyus* Rehd leaves [7] and could function as an inhibitor of α-glucosidase to contro postprandial hyperglycemia during type 2 diabetes [7]. Trilobatin could ameliorate hydrogen peroxide-induced oxidative stress in neuronal PC12 cells [8] and LPS-stimulated macrophages inflammation [9]. However, it has not been reported whether trilobatin can demonstrate anti-oxidative and anti-inflammatory effects in ALI.

Nrf2 (nuclear factor erythroid-2 related factor 2) is a transcriptional factor that can bind to antioxidant response elements of cytoprotective genes to regulate cellular defenses [10]. In addition, Nrf2 can also mediate the activation of nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing 3 inflammasomes [11]. Therefore, Nrf2 is a key regulator of oxidative stress and inflammation-associated diseases, including ALI [12]. Activation of Nrf2 protects against ALI in LPS-induced murine model [13]. Trilobatin has been shown to facilitate the expression of Nrf2 and promote the activation of Nrf2 pathway to repress neurotoxicity and oxidative stress [8, 14].

Collectively, considering the anti-oxidative stress and anti-inflammatory effects of trilobatin, our study was conducted to explore whether trilobatin exerted a potential role on ALI through regulation of Nrf2 pathway. LPS-induced ALI mouse model was firstly established, and the effects of trilobatin on oxidative stress and inflammation in mice model were then

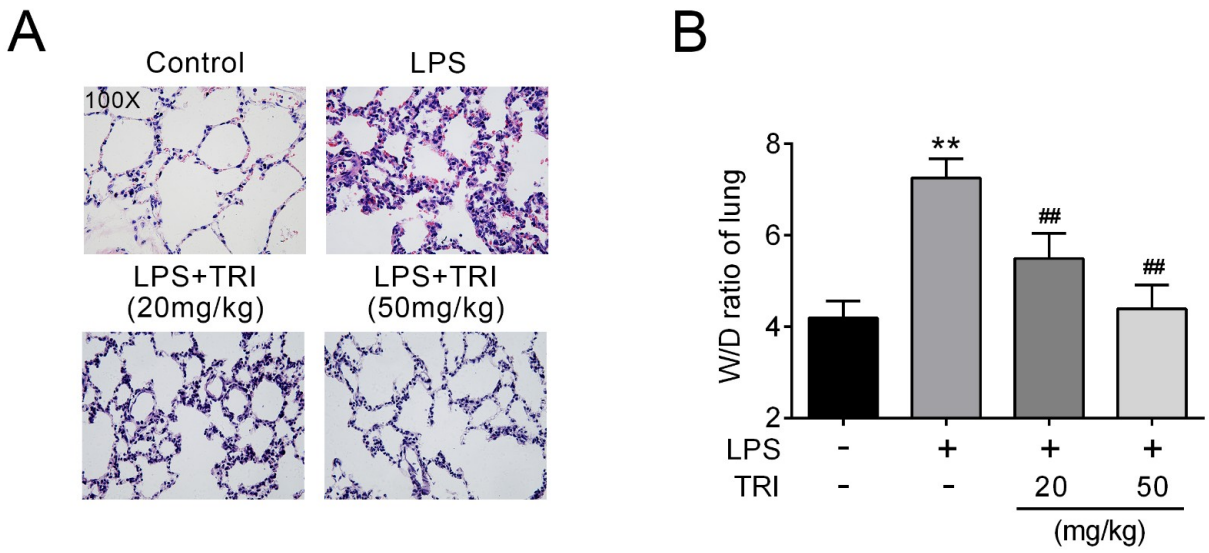

FIGURE 1. Trilobatin alleviated tissue injury in lungs of mice with LPS-induced ALI.
Lung tissues in each group were processed for histological evaluation 12 hours after LPS treatment. W/D ratio in each group was determined 12 hours after LPS treatment.
***p < 0.01 vs. Control, ## p < 0.01 vs. LPS.*

determined. Western blot analysis of proteins involved in Nrf2 pathway further provided a theoretical basis for the future clinical application of trilobatin for the treating ALI.

2. Material and methods

2.1 Mice model with ALI

Experiments involved in animal were approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (Approval no. 20180215). Twenty-four C57 BL/6 male mice (20 - 25 g weight; 6 - 8 weeks old) were acquired from Jackson Laboratory (Bar Harbor, ME, USA), and randomly divided into four groups: Control, LPS, LPS + 20 mg/kg trilobatin and LPS + 50 mg/kg trilobatin. For mice in LPS + trilobatin groups, mice were intraperitoneally administered with 20 or 50 mg/kg trilobatin 1 hour before LPS treatment. For mice in LPS groups, mice were anesthetized with 40 mg/kg pentobarbital sodium, and then intranasally administered with 5 mg/kg LPS in 0.9 % saline according to the study [15]. Mice intranasally administered with saline served as control group. 12 hours after LPS treatment, mice were sacrificed with CO_2 inhalation and then conducted with the analysis.

2.2 Histological analysis

Right lung upper lobe of mice in different groups were excised, immersed in 10% formalin and then embedded in paraffin. After the embedded tissue were sliced as 4 μm thick sections, the samples were deparaffinized, dehydrated and stained with haematoxylin and eosin (H&E) before photographed by microscope (TE2000, Nikon, Tokyo, Japan) according to the study [15].

2.3 W/D ratio

Left lungs of mice in different groups were excised and immediately weighted, and then placed in an 80 °C oven for two days following remove of blood. Weight of the lungs after dehydration was also recorded. The W/D ratio of the lung was then calculated according to the study [16].

2.4 BALF acquisition and analysis

For a collection of BALF, 1 mL of sterile saline was intratracheally injected into right middle lobe of mice in different groups for three times according to the study [15]. The flushing fluid was collected and then centrifuged at 1000 g for 10 minutes. The sedimented cells were then conducted with a hemocytometer and Wright-Giemsa staining to count total cell numbers, neutrophils and macrophages in BALF. The supernatant was used for detection of total protein concentration by BCA protein assay kit (Beyotime, Beijing, China).

2.5 Cytokines measurement and MDA, GSH, CAT, SOD analysis

Left lung lobe of mice in different groups were excised and homogenized in sterile saline. Cytokines (IL-1β and TNF-α) in the supernatant of BALF and lung tissues were determined by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the study [13]. MDA and GSH contents, as well as CAT and SOD activity, in lung tissues, were evaluated by commercially available assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the study [13].

2.6 Western blot analysis

Proteins (30 μg) extracted from lung tissues in different groups were separated and electrophoretically transferred onto a polyvinylidene difluoride membrane (EMD Millipore,

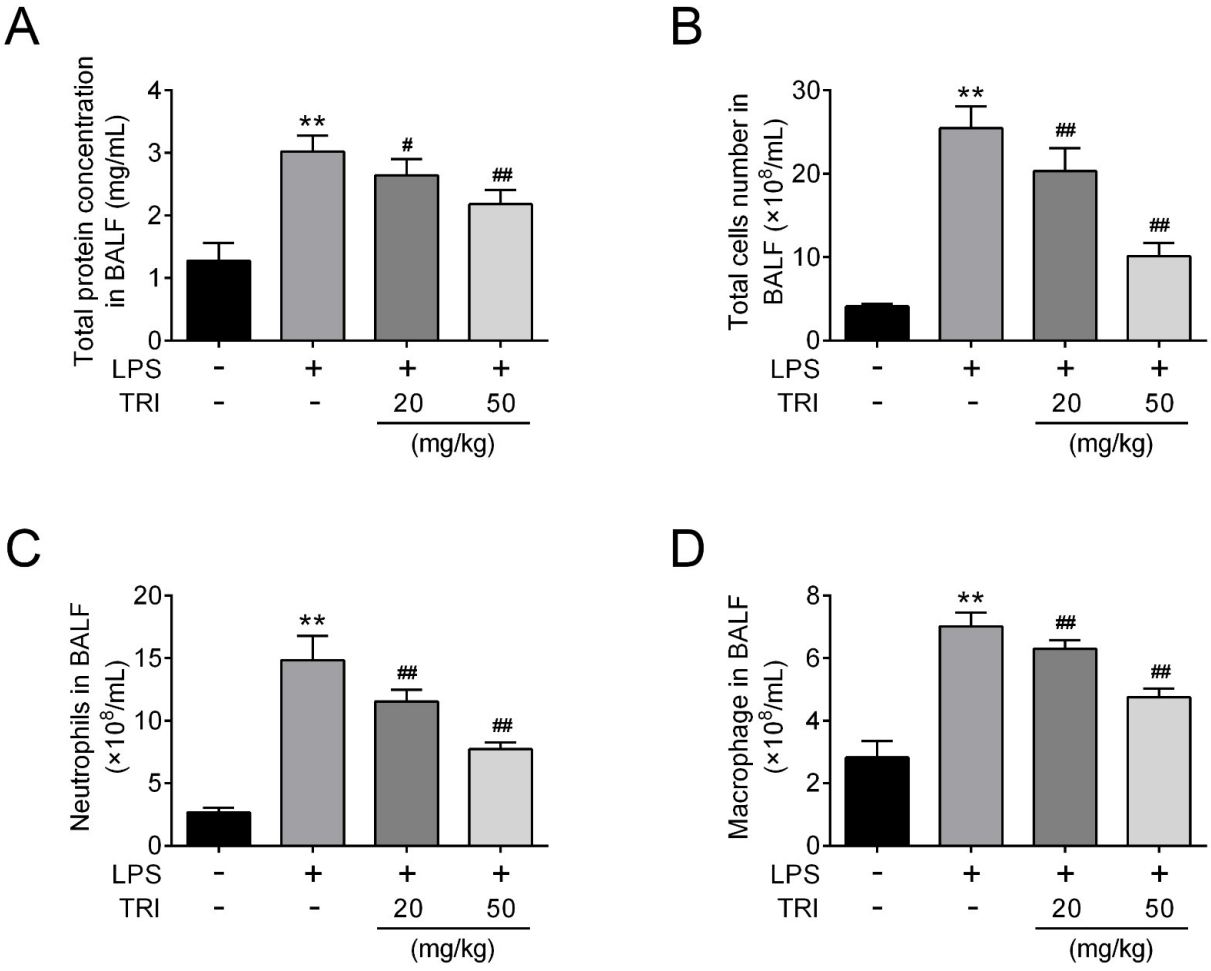

FIGURE 2. Trilobatin repressed protein leakage and reduced cell numbers in BALF of mice with LPS-induced ALI.
Total protein concentrations in BALF from each group were determined 12 hours after LPS treatment.
Total cells number in BALF from each group was determined 12 hours after LPS treatment.
Neutrophils in BALF from each group were determined 12 hours after LPS treatment.
Macrophages in BALF from each group were determined 12 hours after LPS treatment.
*** $p < 0.01$ vs. Control, # $p < 0.05$ ## $p < 0.01$ vs. LPS.*

Bedford, MA, USA). Membranes were incubated with specific primary antibodies against AMPK, p-AMPK (1:1500, Abcam, Cambridge, MA, USA), GSK3β and p-GSK3β (1:2000, Abcam), Nrf2, NF-κB and p-NF-κB (1:2500, Abcam), β-actin (1:3000, Abcam) following blocked with 5% skim milk. Twenty-four hours later, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Abcam), and the protein signals were visualized with ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7 Statistical analysis

All the experiments were repeated at least three times, and data were expressed as the means ± SEM. Following analysis by SPSS19.0 (SPSS Inc, Chicago, IL, USA), statistical differences between experimental groups were evaluated with ANOVA and Tukey's post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 Trilobatin alleviated tissue injury in the lungs of mice with LPS-induced ALI

The current study showed that LPS injection induced lung tissue injuries, including pulmonary congestion and alveolar collapse. While, treatment with trilobatin could attenuate the histological changes in lung tissues treated with LPS (Fig. 1A). Moreover, LPS increased W/D ratio (Fig. 1B), while pretreatment with trilobatin decreased W/D ratio in a dose-dependent manner (Fig. 1B). Taken together, these results indicated that trilobatin alleviated tissue injury in the lungs of mice with LPS-induced ALI.

3.2 Trilobatin repressed protein leakage and reduced cell numbers in BALF of mice with LPS-induced ALI

Protein leakage was increased in BALF following LPS treatment (Fig. 2A). Meanwhile, pretreatment with trilobatin de-

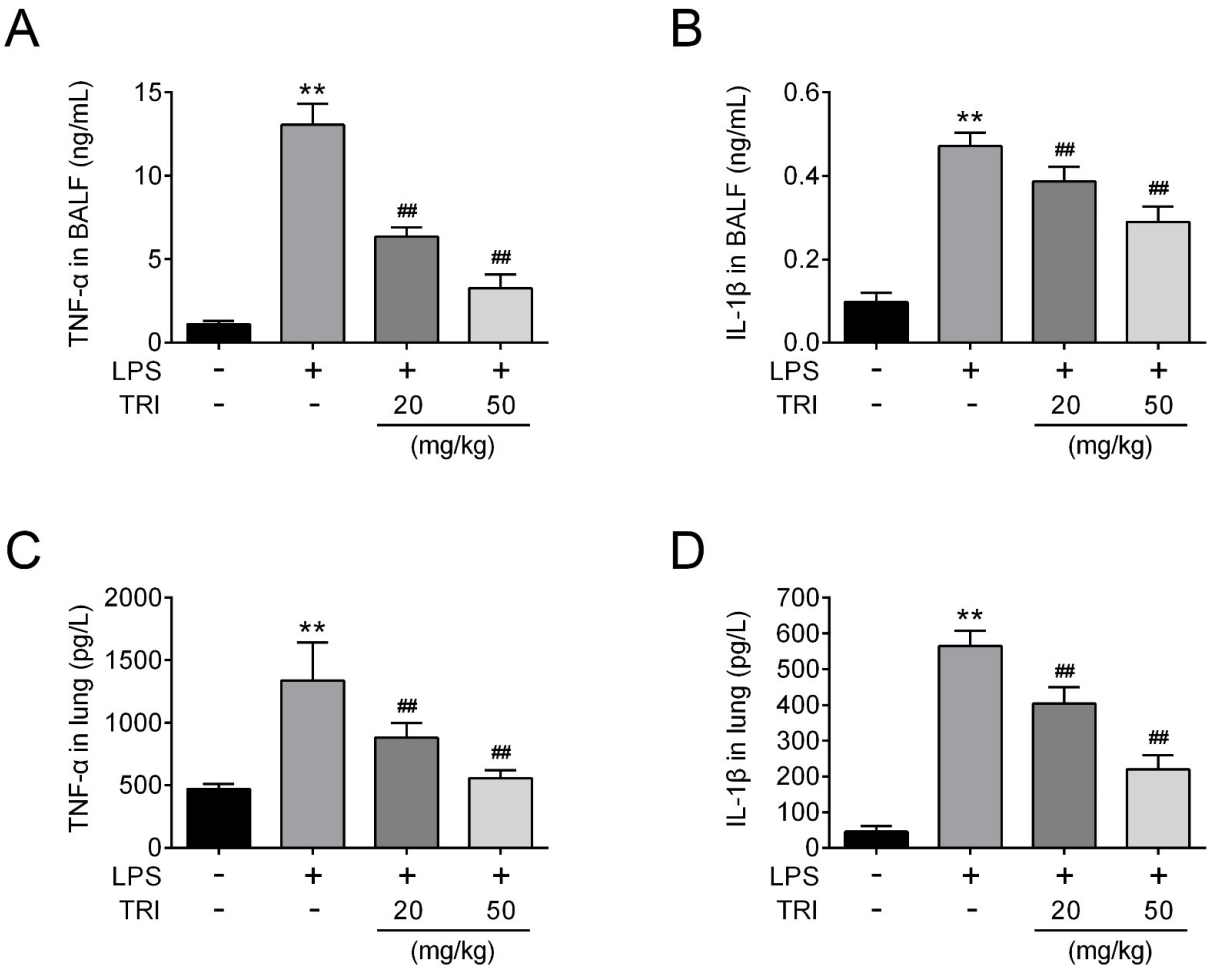

FIGURE 3. Trilobatin repressed inflammatory response in mice with LPS-induced ALI.
Levels of TNF-α in BALF from each group were determined 12 hours after LPS treatment.
Levels of IL-1β in BALF from each group were determined 12 hours after LPS treatment.
Levels of TNF-α in lung tissues from each group were determined 12 hours after LPS treatment.
Levels of IL-1β in lung tissues from each group were determined 12 hours after LPS treatment.
*** $p < 0.01$ vs. Control, ## $p < 0.01$ vs. LPS.*

creased total protein concentration in BALF to prevent protein leakage (Fig. 2A). The total cell numbers (Fig. 2B), neutrophils (Fig. 2C) and macrophages (Fig. 2D) were also increased in BALF following LPS treatment, while pretreatment with trilobatin reduced cell numbers in a dose-dependent manner. These results showed that trilobatin repressed protein leakage and reduced cell numbers in BALF of mice with LPS-induced ALI.

3.3 Trilobatin repressed inflammatory response in mice with LPS-induced ALI

Results showed that trilobatin treatment effectively decreased increase in TNF-α (Fig. 3A) and IL-1β (Fig. 3B) induced by LPS in BALF. Moreover, the increased secretion of TNF-α (Fig. 3C) and IL-1β (Fig. 3D) in lung tissues from mice with LPS treatment were also reduced by trilobatin treatment in a dose-dependent manner, suggesting that trilobatin repressed LPS-induced inflammatory response in ALI mice.

3.4 Trilobatin repressed oxidative stress in mice with LPS-induced ALI

The administration of LPS significantly increased the lung lipid peroxides represented as MDA (5.86 nmol/mg) and remarkablely decreased the content of GSH (220.79 μmol/g), CAT(40.64 U/mg) and SOD activities (29.03 U/mg). However, MDA formation was suppressed and GSH was promoted by trilobatin treatment. In parallel, activities of CAT (Fig. 4C) and SOD (Fig. 4D) were promoted by trilobatin in a dosage-dependent manner, suggesting that trilobatin repressed oxidative stress in LPS-induced ALI mice (Fig. 4A, B, C and D).

3.5 Trilobatin ameliorated LPS-induced ALI in mice via Nrf2 pathway

The result demonstrated that LPS treatment slightly promoted the activities of NF-κB, AMPK, GSK3β and Nrf2, as demonstrated by increase in p-NF-κB, p-AMPK, p-GSK3β and Nrf2 (Fig. 5). Moreover, the promoted activities of AMPK, GSK3β

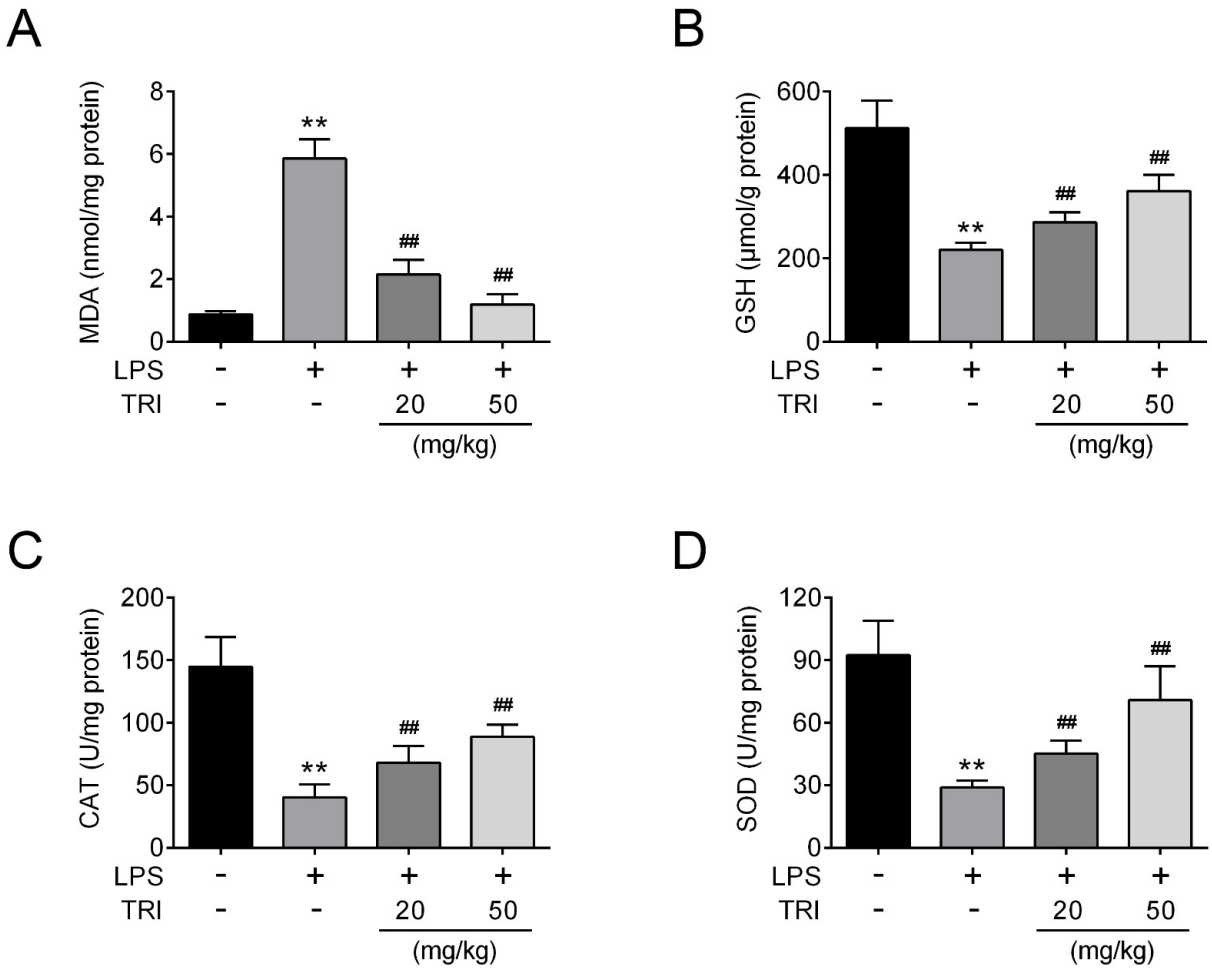

FIGURE 4. Trilobatin repressed oxidative stress in mice with LPS-induced ALI.
Levels of MDA in lung tissues from each group were determined 12 hours after LPS treatment.
Levels of GSH in lung tissues from each group were determined 12 hours after LPS treatment.
Levels of CAT in lung tissues from each group were determined 12 hours after LPS treatment.
Levels of SOD in lung tissues from each group were determined 12 hours after LPS treatment.
*** $p < 0.01$ vs. Control, ## $p < 0.01$ vs. LPS.*

and Nrf2 were strengthened by pretreatment with trilobatin (Fig. 5). However, trilobatin pretreatment decreased protein expression of p-NF-κB thereby down-regulating the activity of NF-κB (Fig. 5), suggesting that trilobatin ameliorated LPS-induced ALI in mice via activation of AMPK/GSK3β-Nrf2 and inactivation of NF-κB pathways

4. Discussion

Protective roles of flavonoids against LPS-induced ALI have been widely reported [17–19]. The flavonoids could repress oxidative stress and inflammation in the ALI model, thus ameliorating the disease [17–19]. Considering that overwhelmed oxidative stress and inflammation are the main pathogenesis of ALI [13]and trilobatin has demonstrated anti-oxidative stress [8] and anti-inflammation [9] roles, the role of trilobatin on ALI was then investigated in this study.

LPS, a compound derived from gram-negative bacteria, could stimulate acute inflammation in tracheal epithelium through up-regulation of proinflammatory cytokines [20]. LPS has been widely used as an inducer of ALI [20]. In this study, LPS administration activated macrophages, promoted neutrophils infiltration, and increased the inflammatory cytokines release (TNF-α and IL-1β), thereby leading to pulmonary edema and the development of ALI. Interestingly, pretreatment with trilobatin attenuated LPS-induced lung injuries with decreased W/D ratio, improved pulmonary congestion and alveolar collapse. Moreover, trilobatin repressed protein and cells leakage, decreased macrophages and neutrophil infiltration, suggesting a protective effect on LPS-induced ALI. In addition, enhancing of neutrophils survival to promote the inflammation, and destruction of the alveolar epithelium through apoptotic pathway also contribute to ALI [21]. Trilobatin was reported to inhibit A β_{25-35}-induced cell apoptosis during Alzheimer' s disease. Whether trilobatin could prevent epithelial cell apoptosis to attenuate ALI remains elusive [22].

LPS could induce initiation of the inflammatory response in macrophages through secretion of cytokines including TNF-α and IL-1β [13]. Inflammatory cells could promote reactive

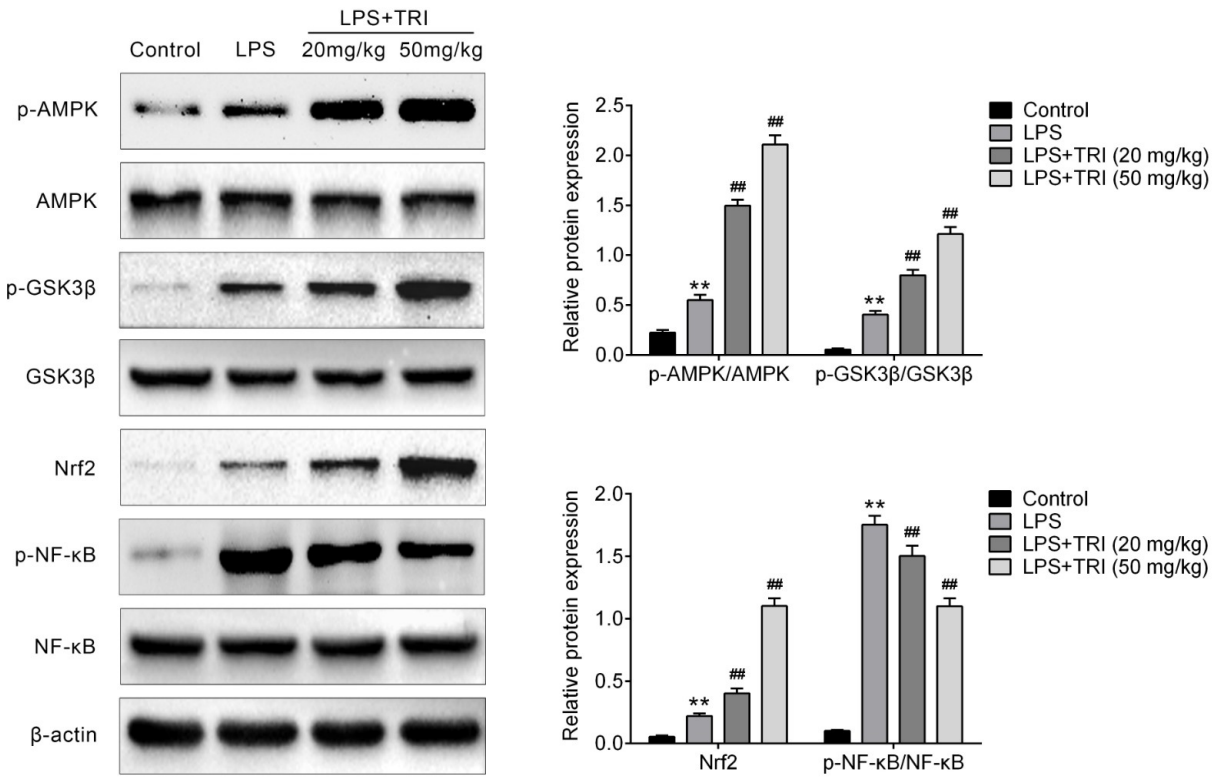

FIGURE 5. Trilobatin ameliorated LPS-induced ALI in mice via Nrf2 pathway.
*Levels of AMPK, p-AMPK, GSK3β, p-GSK3β, Nrf2, NF-κB and p-NF-κB in lung tissues from each group were determined 12 hours after LPS treatment. ** $p < 0.01$ vs. Control, ## $p < 0.01$ vs. LPS.*

oxygen specie accumulation to exaggerate the oxidative stress, and LPS-induced oxidative stress could also aggravate inflammation [13]. Therefore, inflammation and oxidative stress formed a vicious cycle to stimulate the development of ALI, and the way to prevent oxidative stress and inflammation represent a classic strategy for ALI. Previous study has shown that trilobatin could inhibit mitochondrial reactive oxygen species homeostasis [8], and suppressed LPS-induced inflammatory response by decreasing TNF-α, IL-6 and IL-1β [9]. Results in this study indicated that pretreatment with trilobatin inhibited secretion of TNF-α and IL-1β, decreased production of MDA and increased anti-oxidative enzymes (GSH, CAT and SOD), thus demonstrating anti-inflammation and anti-oxidative stress effects against ALI.

AMPK is an energy sensor during cellular metabolism which can inactivate GSK3β, thereby promoting Nrf2 nuclear translocation, and Nrf2 is a central coordinator in inflammatory and oxidative pathways [23]. Nrf2 could attenuate LPS-induced inflammation and oxidative stress during ALI [23]. Data from this study revealed that treatment with trilobatin activated AMPK with increased p-AMPK, then inactivated GSK3β through up-regulation of p-GSK3β, and finally increased Nrf2. Trilobatin was reported to prevent oxidative damage by the activation of AMPK/Nrf2 pathway to increase Sirt3 [8], and protect neurotoxicity through Nrf2-mediated heme oxygenase-1 and NAD (P)H quinone dehydrogenase 1 [14]. The downstream target of Nrf2 during trilobatin-mediated ALI needs to be further investigated. Additionally, anti-inflammatory pathway (Nrf2) and proinflammatory pathway (NF-κB) regulate inflammatory response during development of ALI [24]. Trilobatin could suppress NF-κB pathway to ameliorate LPS-induced inflammatory response [9]. Pretreatment with trilobatin also decreased p-NF-κB to prevent inflammation in LPS-induced ALI. The clinical application of trilobatin on patients with ALI will be an urgent need to treat the disease.

5. Conclusion

This study demonstrated that trilobatin effectively prevented oxidative stress and inflammation in ALI, which largely depends on the activation of AMPK/GSK3β-Nrf2 pathway and inactivation of NF-κB pathway. The meaning results provided a potential theory for application of trilobatin in the prevention of ALI.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest to disclose.

STATEMENT OF INFORMED CONSENT

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

ETHICS APPROVAL

Ethical approval was obtained from t the Ethics Committee of Nanfang Hospital, Southern Medical University (Approval no. 20180215).

AUTHOR CONTRIBUTIONS

Hai Zhong and Long Hao designed the study, supervised the data collection, Xiang Li analyzed the data, interpreted the data, Chunjing Wang and Xu Wu prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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